PROTOCOL

Three-dimensional cell culturing by magnetic levitation

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Recently, biomedical research has moved toward cell culture in three dimensions to better recapitulate native cellular environments. This protocol describes one method for 3D culture, the magnetic levitation method (MLM), in which cells bind with a magnetic nanoparticle assembly overnight to render them magnetic. When resuspended in medium, an external magnetic field levitates and concentrates cells at the air-liquid interface, where they aggregate to form larger 3D cultures. The resulting cultures are dense, can synthesize extracellular matrix (ECM) and can be analyzed similarly to the other culture systems using techniques such as immunohistochemical analysis (IHC), western blotting and other biochemical assays. This protocol details the MLM and other associated techniques (cell culture, imaging and IHC) adapted for the MLM. The MLM requires 45 min of working time over 2 d to create 3D cultures that can be cultured in the long term (>7 d).

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INTRODUCTION

In living tissue, cells exist in 3D microenvironments with intricate cell-cell and cell-matrix interactions and complex transport dynamics for nutrients and cells[1–5]. Although standard 2D or monolayer cell culture has been crucial for the development of modern biology, it inadequately recreates the natural environment within which cells reside. This lack of fidelity to the native tissue can be a severe limitation in many situations, including drug screening for toxicity and efficacy[6–9]. This limitation has led to the development of *in vitro* 3D cell culture techniques designed to provide a more physiologically relevant cellular environment that could potentially improve basic research and the drug discovery process[1–5,10–12].

We recently developed the MLM to produce 3D cell cultures[13]. In this method, a magnetic nanoparticle assembly comprising gold nanoparticles, iron oxide and cell-adhesive peptide sequences is delivered to cells in 2D culture to render these cells magnetic[13–16]. When unattached and suspended in liquid, the cells can be manipulated with the external application of magnetic forces[13]. In particular, cells in a Petri dish or in a multiwell plate can be levitated to the air-liquid interface[13]. At the air-liquid interface, the cells interact and aggregate together into larger structures while synthesizing ECM proteins like collagen, fibronectin and laminin[17]. Overall, the MLM can be used to create 3D cell cultures with physiologically relevant ECM. Furthermore, the particular components of this method are nontoxic, do not affect proliferation and do not induce an inflammatory response by the cultured cells[13,17]. The protocol describes the creation of 3D cultures using the MLM, as well as other common techniques adapted to the use of magnetically levitated 3D cultures, including medium replacement, imaging, handling and IHC. We also show typical results seen when applying the MLM to various cell types.

Applications of the method

The MLM generally uses the magnetic nanoparticle assembly to promote delivery of the magnetic nanoparticles, making the MLM broadly applicable to most cell types[13,17,18]. Indeed, the MLM has been successfully used to make 3D cultures with all cell types tested to date, including cell lines, stem cells and primary cells[13,17–22] (**Table 1**). The most basic application of the MLM is to culture 3D cell cultures under different biochemical or environmental conditions, and then analyze them using common biological research techniques, such as IHC[17,18] and western blotting[19]. The ability to magnetically manipulate 3D cultures also allows for fine spatial control and more complex environments. For example, the MLM was used to create an invasion assay between two separate cultures of human glioblastoma and normal astrocytes to investigate the mechanisms of glioblastoma invasion[13,19]. The MLM has also been used to create coculture models of the bronchiole[17] by sequentially assembling multiple 3D cultures in a layered fashion. In addition, the MLM has been used to differentiate stem cells in 3D; 3T3-L1 preadipocytes and adipose stem cells were differentiated into adipocytes and formed into a vascularized adiposphere in coculture with endothelial cells[18,21]. These 3D cultures are also scalable in size, so that cultures can not only be created in 96-well plates as described in this protocol but also larger cultures can be constructed in six-well plates or Petri dishes. Overall, the MLM is a versatile tool for performing basic and complex experiments in representative 3D environments.

Comparison with other methods

There are many systems for 3D cell culture, including protein gel substrates[23–25], synthetic polymer scaffolds[26,27] and spheroids[28,29]. However, these methods are costly, often involving extensive fabrication and time-consuming analysis. For example, some protocols for spheroids take 3–4 d for spheroid formation[28,29], whereas the MLM takes about 16 h to form 3D cultures. The MLM also creates 3D cultures without an artificial protein substrate, and, in fact, can synthesize ECM during formation, as demonstrated when 3D cultures of human pulmonary fibroblasts and smooth muscle cells alone produced and extruded laminin within hours of levitation[17]. The MLM also does not require any specialized media or a minimum serum concentration. In general, 3D cultures are cultured using the preferred medium for 2D cultures. For example, bronchial epithelial cells have been assembled in 3D without serum, owing to their sensitivity in 2D,

TABLE 1 | Cell types that have been magnetically levitated into 3D culture.

	Name	Animal	Type
Cell lines	HEK293	Human	Embryonic kidney
	MDA-231	Human	Mammary epithelial
	MCF-10A	Human	Mammary epithelial
	LNCaP	Human	Prostate epithelial
	A549	Human	Alveolar epithelial
	HepG2	Human	Hepatocyte
	3T3-L1	Mouse	Fibroblast[18]
	bEnd.3	Mouse	Brain endothelial[18]
	H-4-II-E	Rat	Hepatoma
	U251-MG	Human	Glioblastoma[13,19]
Primary cells		Human	Astrocyte[13,19]
		Human	Pulmonary endothelial[17]
		Human	Type II alveolar epithelial
		Human	Bronchial epithelial[17]
		Human	Tracheal smooth muscle[17]
		Human	Pulmonary fibroblasts[17]
		Human	Umbilical vein endothelial
		Human	Chondrocytes
		Human	Aortic vascular smooth muscle[22]
		Porcine	Aortic valvular interstitial
		Porcine	Aortic valvular endothelial
Stem cells		Human	Neural stem cells[13]
		Human	Mesenchymal stem cells
		Human	Dental pulp stem cells
		Human	Adipose stem cells[18]

whereas pulmonary fibroblasts need serum to grow in 3D, just as they require it in 2D (ref. 17). Thus, the MLM is a simpler tool for creating representative 3D cell culture environments when compared with other methods.

Experimental design

In this protocol we describe how to apply the MLM to create 3D magnetically levitated cultures that can replace 2D cultures. Such cultures can be used for various applications, such as investigating the dose-dependent effects of a particular drug on a particular cell type of interest. Cells are cultured in advance to confluence in 2D, but on the day before the experiment is to start, the cells are incubated with a magnetic nanoparticle assembly overnight to allow for cell attachment to the magnetic nanoparticles (Steps 1–4). The next day, the cells are detached and resuspended in medium in a 96-well plate (Steps 5–8). A magnetic drive is placed atop the well plate to levitate the cells to the air-liquid interface, where the cells aggregate and interact to form larger 3D structures (Step 9). Once the structures are fully formed and become competent (Step 10), compounds can be added to each well at specific concentrations. At a specific time point, the 3D cultures can then be assayed similarly to 2D cultures with tests such as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[17], IHC[17,18] (Steps 11–23) or western blotting[19]. Other, more complex experiments including cocultures[17,18] can be conducted as well with the MLM. In all, magnetically levitated 3D cultures can replace 2D cultures with minimal adaptation to existing protocols.

Limitations

The magnetic nanoparticles have been shown to not affect cell proliferation and metabolism or induce an inflammatory response[13,17]. By 8 d, cells will detach from and grow without the magnetic nanoparticles, which are retained within the culture[13]. Similarly, exposure to the magnetic field (30–500 G) has no major effect on cell proliferation, metabolism or inflammatory response[13,17], although magnetic fields of higher strength (800–4,000 G) have been shown to influence cell behavior[30–32]. In addition, the presence of iron oxide within the magnetic nanoparticle assembly will color the 3D culture brown. Although this can enhance contrast for some imaging diagnostics, the dark color may also be viewed as a disadvantage for some applications. In particular, the use of colorimetric markers that are brown, such as IHC with 3,3-diaminobenzidine, is limited. However, common colorimetric assays, such as the MTT assay, have been successfully used on cells incubated with magnetic nanoparticles[17], and fluorescence is not hindered by them either[17,18]. There is also possibility of cell loss due to the incomplete attachment of cells to the magnetic nanoparticles during incubation that could adhere to the bottom of the plate rather than levitate, but this limitation can be addressed with the use of ultra-low-attachment multiwell plates.

MATERIALS

REAGENTS

- ▲ **CRITICAL** All reagents can be purchased from other distributors and manufacturers than those listed.
- NanoShuttle (Nano3D Biosciences, cat. no. 005-NS; or see Souza and colleagues[13,15,16] for directions on how to fabricate magnetic nanoparticles yourself)
- Cells of interest
- Cell culture medium ▲ **CRITICAL** Use cell culture medium specific to the cell type to be turned into 3D. There is no minimum serum concentration required.
- Trypsin-EDTA, 1× (Corning, cat. no. 25-053-CI)
- Trypsin neutralizer solution (Invitrogen, cat. no. R-002-100)
- Ethanol (Sigma-Aldrich, cat. no. E7023)
- Phosphate buffered saline, pH ~7.4 (PBS Sigma-Aldrich, cat. no. P5368)

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- Paraformaldehyde, 4% (wt/vol) (PFA; Electron Microscopy Sciences, cat. no. 15735-100)
- Triton X-100 (TX-100; Sigma-Aldrich, cat. no. T8787)
- Blocking buffer ▲ CRITICAL Choose a blocking serum that does not interfere with the primary and secondary antibodies used.
- Primary antibody (use an antibody for specific antigen of interest)
- Secondary antibody (use an antibody for the specific animal of the primary antibody)
- DAPI (KPL, cat. no. 71-03-01)

EQUIPMENT

- Cell culture flask, 75 cm^2
- Pipette gun
- Pipette, 5 ml
- Incubator (37 °C, 5% CO_2)
- Laminar flow cabinet
- Refrigerator (4 °C)
- Freezer (−20 °C)
- Pipettor, 200 μl
- Pipette tip, 20–200 μl
- Conical tubes, 15 ml
- Hemocytometer or cell counter
- Magnetic drive consisting of an array of 96 neodymium magnets (Nano3D Biosciences, cat. no. 009-96WD)
- Plastic lid insert for 965-well plates (Nano3D Biosciences, cat. no. 009-96LID)
- Ultra-low-attachment plates, 965 well (Corning, cat. no. 3474)
- Pasteur pipettes
- Inverted microscope
- MagPen three-pack (comes with 0.125-inch inner diameter (i.d.) × 0.200-inch outer diameter (o.d.) Teflon pen, 0.125-inch o.d. rod magnet; Nano3D Biosciences, cat. no. 010-MPT)
- Aluminum foil
- Coverslips

REAGENT SETUP

NanoShuttle NanoShuttle can be stored at 4 °C for 1 year. ! CAUTION Do not freeze it.

TX-100 Prepare TX-100 in a 0.2% (vol/vol) solution in ultrapure H_2O. The solution can be stored at 4 °C for years.

Blocking buffer Prepare blocking buffer by diluting blocking serum at a 1% (vol/vol) concentration in PBS. The stock solution can be stored according to the manufacturer's directions.

Primary and secondary antibodies Dilute the primary and secondary antibodies according to the manufacturer's recommended dilutions in PBS. Do not store working solutions. Freshly prepare the working solutions for every IHC staining session. Keep secondary antibody stock and working solutions away from light. The stock solutions can be stored according to the manufacturer's directions.

DAPI Prepare DAPI and dilute it according to the manufacturer's recommended dilutions. Do not store the working solution. Freshly prepare the working solution for every IHC staining session. Keep the stock and working solutions away from light. The stock solution can be stored at −20 °C for 1 year.

PROCEDURE

Incubation of cells with magnetic nanoparticles ● TIMING working time 20 min, incubation time 5–16 h

▲ CRITICAL Perform the following steps under sterile conditions using the recommended cell culture supplies for the specific cell type.

1| Culture cells in 2D to ~80% confluence using standard cell culture procedures and supplies for the specific cell type.

2| Prepare the magnetic nanoparticle assembly by removing it from the refrigerator and thawing it at room temperature (20–25 °C) for about 15 min.

▲ CRITICAL STEP Ensure that the magnetic nanoparticles are homogenized before use, which results in an even brown color throughout the solution. If the magnetic nanoparticles are not homogenized, they must be homogenized in the vial in a sterile environment by mixing with a pipette at least ten times.

? TROUBLESHOOTING

3| Add the magnetic nanoparticles directly to the cells and medium in the flask at a recommended concentration of 8 μl cm^{-2} of culture area. Gently tilt the flask back and forth to evenly distribute the nanoparticles around the flask. The medium will appear slightly darker because of the brown color of the iron oxide.

▲ CRITICAL STEP Before experimentation, optimize for cell binding to the nanoparticles by varying the volume of magnetic nanoparticles added.

4| Put the flask back into an incubator to let the cells incubate and attach to magnetic nanoparticles for at least 5 h to overnight.

! CAUTION Longer incubation times will result in the nanoparticles detaching from the cells.

? TROUBLESHOOTING

Creating 3D cultures with MLM in 96-well plates ● TIMING 25 min

5| Aspirate the medium from the flask and detach cells by incubation with trypsin-EDTA solution for 2–5 min. Concomitantly, sterilize the well plate, magnetic drive and the lid insert with 70% (vol/vol) ethanol and bring them into the sterile environment. Once the cells are detached, add medium with serum at four times the volume of trypsin to neutralize the trypsin, and then transfer the solution into a conical tube. For more sensitive cell types, use the detachment protocol

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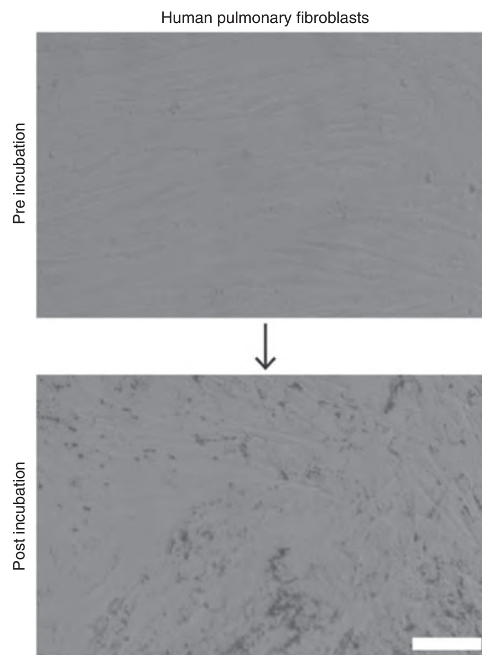
Figure 1 | Human pulmonary fibroblasts before and after incubation with magnetic nanoparticles. The cells maintained their morphology but became peppered with nanoparticles after incubation. Scale bar, 100 μm.



for the specific cell type. When settled or centrifuged, the cells should appear brown in color, and cell suspensions in medium should appear darker than usual (**Fig. 1**).
! CAUTION Do not autoclave the magnetic drive beforehand. Magnets will demagnetize when exposed to high temperatures. The lid insert can be autoclaved, but take care that you keep it flat. Sterilization with 70% (vol/vol) ethanol is sufficient.
▲ CRITICAL STEP Before detachment, check the binding of cells to the magnetic nanoparticles under a microscope. Cells should appear peppered with the nanoparticles (**Fig. 1**).
? TROUBLESHOOTING

6| Count the number of cells in suspension using either a hemocytometer or cell counter.

7| Calculate the cell numbers and medium volumes needed to create 3D cultures. Typical cell numbers and medium volumes are 500–5,000 cells in 50–75 μl in 96-well plates (**Fig. 2**). 3D cultures can also be created in larger-well plates, such as 24-well plates. See **Supplementary Figure 1** for information on how to form 3D cultures in a 24-well plate.
! CAUTION Too much medium in the well will bring 3D cultures closer to the magnet, which could result in cells escaping the medium rather than levitating in it.
▲ CRITICAL STEP Optimize for the size and competence of 3D cultures by varying cell number and medium volume before experimentation.
▲ CRITICAL STEP If you are using larger plates, such as 24-well plates, optimize the cultures by varying the cell number and medium volume.

8| Add the desired medium volume with the desired cell concentration to the wells in the plate. Gently agitate the well plate to evenly distribute the medium in the well (**Fig. 3**).
▲ CRITICAL STEP Use flat-bottom, ultra-low-attachment plates for maximum levitation efficiency.
? TROUBLESHOOTING

9| Close the well plate in the following order: first, close the lid insert, then the magnetic drive and finally, the well-plate lid. Move the well plate to the incubator. 3D structures will begin to form within 15 min–1 h. These cultures should appear dense and brown, and should levitate at or slightly below the air-liquid interface (**Fig. 4**).
! CAUTION Keep the plate flat during handling, as tilting the plate could bring the 3D culture closer to the magnet, where it could escape the medium and attach to the lid insert.
? TROUBLESHOOTING

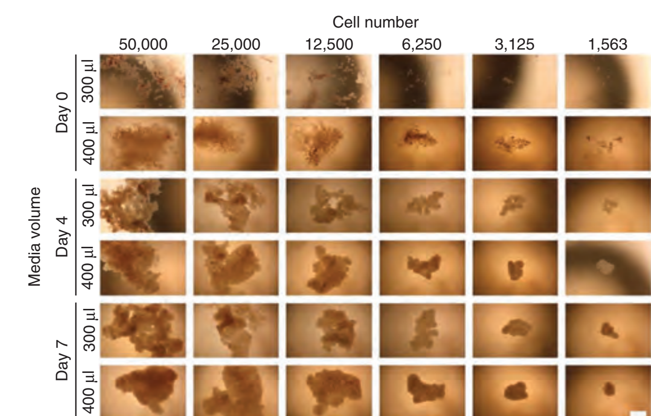


Figure 2 | Magnetically levitated 3D cultures of A549 cells. Shown are different cell numbers (1,000–50,000 cells) and medium volumes (300 or 400 μl) after 0, 4 and 7 d. Scale bar, 500 μm.

Culturing 3D magnetically levitated cultures

10| Maintain the 3D cultures in an incubator (37 °C, 5% CO_2) for the length of the experiment. Medium should be replaced (option A) at regular intervals depending on the protocols for the specific cell type and experiment. 3D culture growth should be monitored by imaging regularly (option B). These cultures can also be transferred between plates (option C) for imaging or staining. When you wish to

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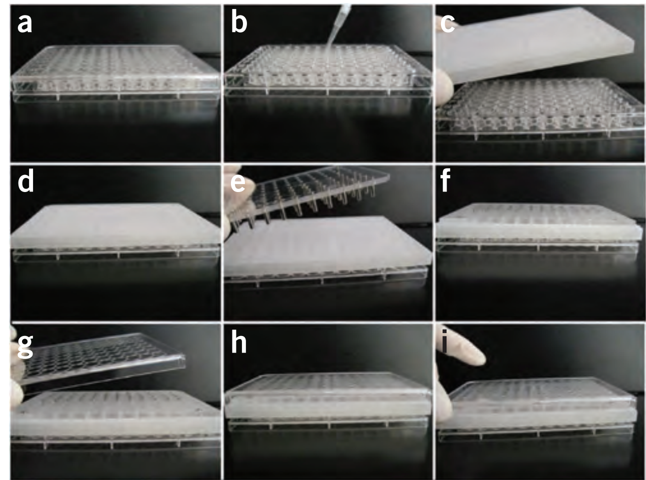

Figure 3 | Magnetic levitation in 96-well plates. (**a**,**b**) First, take a 96-well plate (**a**) and add 50–75 µl of medium with cells to each well (**b**). (**c**–**h**) Next, cover the plate with a 96-well white lid insert (**c**,**d**), 96-well magnetic drive (**e**,**f**) and lid (**g**,**h**). (**i**) The plate lid can then be annotated and the plate can be transferred into an incubator.

fix the cells and perform IHC, proceed to Step 11.

(A) Replacing medium in well plates with 3D cultures ● TIMING **5 min**

(i) Sterilize the outside of the well plate with 70% (vol/vol) ethanol and bring it into a sterile environment. Open the well plate, and move the lid insert, magnetic drive and lid away from the plate, with each component turned upward.

(ii) Take the magnetic drive and move it underneath the well plate with the magnets facing upward. The 3D cultures should be attracted by the magnet and move to the bottom of the well plate. Position the plate such that the magnets are off-center within the wells and that there is sufficient space to remove medium without damaging the 3D culture (**Fig. 5**).

▲ CRITICAL STEP Ensure that the cultures are at the bottom of the wells.

? TROUBLESHOOTING

(iii) Aspirate the medium out of the wells and gently replace the medium.

? TROUBLESHOOTING

(iv) Remove the magnetic drive from underneath the plate, and then cover the plate in the order described in Step 9. Move the plate back into the incubator.

(B) Imaging 3D cultures in a well plate with an inverted microscope ● TIMING **5 min**

(i) Follow Step 10A(i) to open the plate in a sterile environment (**Supplementary Fig. 2**).

(ii) Replace the lid insert and lid atop the well plate. Remove the plate from the sterile environment and move it to a microscope stage.

! CAUTION Keep the plate flat, as rough handling could disrupt the 3D culture.

▲ CRITICAL STEP The lid insert is translucent, so cultures can be viewed without the need to remove the lid insert from the plate. If the culture is difficult to image with the lid insert on, remove the lid insert in a sterile environment and follow Step 11.

? TROUBLESHOOTING

(iii) Return the plate to the sterile environment. Repeat Step 9 to close the plate and move the plate back into the incubator.

(C) Handling and transferring 3D structures with a Teflon pen ● TIMING **5 min**

(i) Sterilize the Teflon pen and rod magnet with 70% (vol/vol) ethanol and move them into a sterile environment.

! CAUTION Do not autoclave the rod magnet. Sterilization with 70% (vol/vol) ethanol is sufficient. The Teflon pen can be autoclaved.

(ii) Assemble the Teflon pen by inserting the rod magnet. The pen can be handled with gloved hands or plastic forceps.

! CAUTION Do not use forceps made of magnetic metals, which would attract the rod magnet within the pen.

? TROUBLESHOOTING

(iii) Repeat Step 10A(i) to open the plate of 3D cultures in a sterile environment.

! CAUTION Keep the magnetic drive a good distance away from the working area to prevent it from interfering with the Teflon pen.

(iv) With the Teflon pen facing downward, reach into the well to pick up the 3D culture. The 3D culture should be attracted and attached to the Teflon pen. Lift the pen from the well plate and remove the rod magnet from the pen. The 3D culture should stay attached to the Teflon pen (**Fig. 6**).

? TROUBLESHOOTING

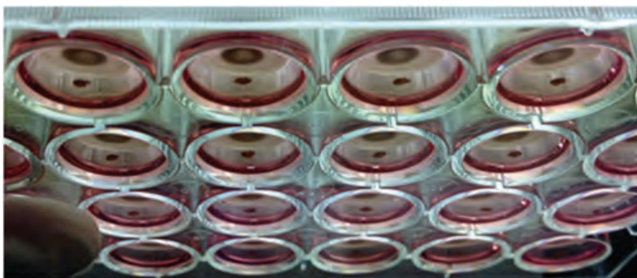

Figure 4 | Magnetically levitated 3D cultures levitating in medium in a 24-well plate. Note the brown color of the cultures.

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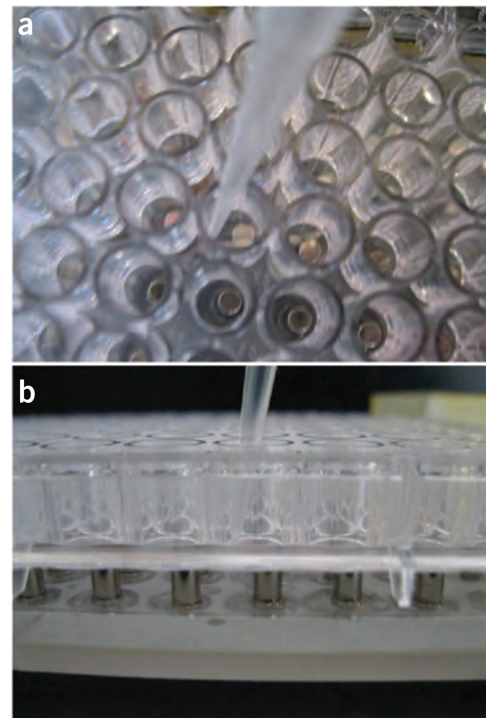

Figure 5 | Replacing medium with magnetically levitated 3D cultures. (**a**) Place the 96-well plate on a magnetic drive, but leave it off-center so that cells are held on one side of the well. (**b**) Medium can then be replaced in the well away from the culture without damaging it.

(v) With the magnets facing upward, place the magnetic drive underneath the new well plate to which the 3D culture is being transferred.

(vi) Lower the pen with the 3D culture still attached to the bottom of the new well. The magnetic drive should attract the 3D culture off the pen to bottom of the well (**Fig. 6**).
? TROUBLESHOOTING

(vii) Gently add medium to the well.

(viii) Repeat Step 10A(iv) to close the plate and move the plate into the incubator.

Fixing 3D cultures for IHC ● TIMING working time 5 min, fixation time 15 min–4 h

11| Follow Step 10A(i–iii) to replace the medium with PBS. Aspirate and then wash with PBS once more.

12| Remove the magnetic drive from underneath the plate.

13| Add your preferred fixative, such as 4% (wt/vol) PFA, and fix the cultures for 15 min–4 h at room temperature depending on the size of the culture (small cultures will require less fixation time).
▲ CRITICAL STEP This and the following steps can be performed in a nonsterile environment.

14| After fixation, repeat Step 11 to remove the fixative and wash the 3D culture with PBS twice.
■ PAUSE POINT If you wish to store the fixed cultures to use later, add PBS to each well and close the plate with the plate lid. Wrap the plate in paraffin film and store it at 4 °C for later use. Fixed cells can be stored at 4 °C for several months.

Whole-mount IHC of 3D cultures with fluorescence ● TIMING working time 45 min, incubation time 4–16 h

15| Follow Step 10C to transfer the fixed 3D culture to a new 96-well plate. Leave the magnetic drive underneath the plate for now.
▲ CRITICAL STEP Although this protocol describes the IHC staining of whole-mounted 3D cultures, these cultures should be treated as a tissue that can also be frozen or paraffin-embedded for sectioning using routine protocols[17]. Follow standard protocols for processing, embedding, sectioning and staining tissue.

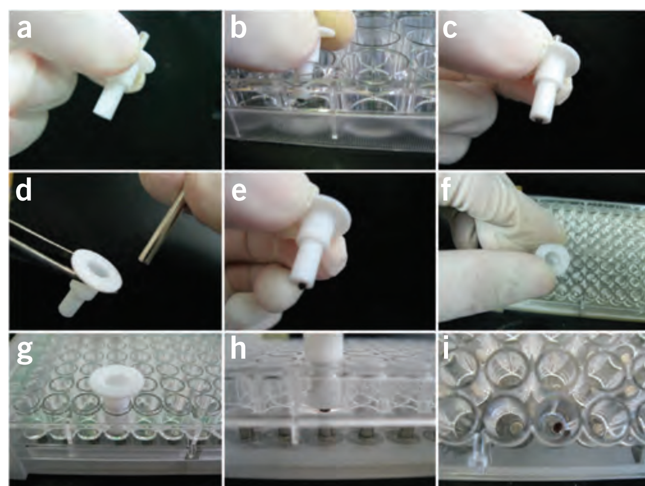

Figure 6 | Transferring 3D cultures from a 24-well plate to a 96-well plate using the Teflon pen. (**a**,**b**) Assemble the pen (**a**) and use it to pick up the 3D culture from the 24-well plate (**b**). (**c**,**d**) With the culture attached to the pen (**c**), remove the magnet from the pen (**d**). (**e**) The 3D culture should still stay on the pen. (**f**,**g**) Move to the 96-well plate with a 96-well magnetic drive underneath it (**f**) and place the pen in a well (**g**). (**h**,**i**) The magnet underneath the plate (**h**) should pull the 3D culture down to the bottom of the well plate (**i**).

16| For intracellular antigens, permeabilize the cell membrane with 0.2% (vol/vol) TX-100 for 15 min. Thereafter, repeat Step 11 to remove the TX-100 solution and wash the culture with PBS five times. For this and the following steps, the 3D cultures can be stained in the same well for the remainder of the experiment either with the magnetic drive underneath the plate to maintain culture structure, or

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without the magnetic drive to allow for solutions to penetrate underneath cultures. The 3D cultures can also be transferred to a new well after incubation with each solution (follow Step 10C).
? TROUBLESHOOTING

17| Add blocking buffer to the culture and incubate it for 1 h at room temperature. Blocking will prevent nonspecific antibody binding. Bring the plate back onto the magnetic drive, and for experimental wells, remove the blocking buffer.

18| For experimental wells, remove the blocking buffer by following Step 11. Add the primary antibody solution to incubate it for either 1 h at 37 °C or overnight at 4 °C. Incubate the negative control wells with the blocking buffer.
▲ CRITICAL STEP Perform IHC using the manufacturer's recommended dilutions for the specific antigen of interest.

19| In all wells, repeat Step 11 to aspirate all solutions and wash the cultures twice with PBS. Remove the magnetic drive and add the fluorescently labeled secondary antibody solution to incubate for 1 h at room temperature.
! CAUTION From this step onward, cover the plate in aluminum foil to prevent photobleaching of the fluorescent tags.
▲ CRITICAL STEP Check that the target animal of the secondary antibody corresponds with that of the primary antibody and does not match the animal source of the blocking buffer.

20| Repeat Step 11 to aspirate the secondary antibody solution from the wells, and wash the cultures twice with PBS. Add DAPI to counterstain the nuclei, and then incubate the cells for 15 min at room temperature.

21| Repeat Step 11 to aspirate the DAPI solution and wash the cultures twice with PBS. Leave the culture in PBS and remove the magnetic drive.
? TROUBLESHOOTING
■ PAUSE POINT If you wish to store the stained culture to image later, add PBS to each well and close the plate with the plate lid. Wrap the plate in paraffin film and store it at 4 °C for later use. Depending on the fluorophores used, stained cultures can be stored at 4 °C for several months. Refer to the manufacturer's instructions for storage of stained cultures.

Transferring 3D cultures from plates to coverslips for microscopy ● TIMING 5 min
22| On a magnetic drive with its magnets facing upward, place a coverslip on top of the magnets (**Supplementary Fig. 3**).

23| From the well plate, pick up the sample using the Teflon pen as described in Step 10C. Deposit the 3D culture directly onto coverslip and proceed to imaging the culture.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
2	Magnetic nanoparticles appear separated	Magnetic nanoparticles has settled at the bottom of the vial	Homogenize the nanoparticles by pipetting up and down ten times
4	Cells do not appear to fully bind with magnetic nanoparticles, and there are nanoparticles still floating in medium	Binding with the nanoparticles will vary in efficiency among cell types	Cells will not always fully bind with magnetic nanoparticles and will appear peppered. These cells will still levitate. Add fewer nanoparticles if there is an excessive amount
		Cells may have incubated with the nanoparticles for too long	Incubate cells with nanoparticles at most overnight
5	Cells are taking longer than 5 min to detach	Cells are strongly adhered to the substrate	Before adding trypsin, wash the flask with PBS
	Cells are sensitive to serum	Cells may differentiate with serum	Use a trypsin-neutralizing solution in lieu of medium with serum to stop enzymatic activity. Centrifuge the cells down and resuspend in medium before counting

(continued)

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TABLE 2 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
8	Cells appear to be attaching to the bottom of plate	Incubated cells may not be bound to the magnetic nanoparticles or are weakly bound	Use ultra-low-attachment plates to prevent cells from adhering and at least collect those cells that are weakly bound
9	Cells escape the medium and attach to the lid insert	Too much volume of medium	Keep medium volumes within the recommended range in the protocol
		Plate tilted too far	When handling the plate, always keep the plate flat to prevent the medium from tilting
	Levitated cells appear to spread out	Cells have not been levitated for enough time	Levitate the cells for a longer amount of time and carefully monitor the formation of the 3D culture
	The lid insert appears warped and does not fit correctly	The lid insert has been modified, likely by heat	Replace the lid insert and do not autoclave the magnets. Sterilization with 70% (vol/vol) ethanol should be sufficient
10A(ii)	Some of the culture is breaking off during aspiration	Medium is being aspirated too closely to the culture	Position the magnetic drive under the plate so that the magnet is off-center within the plate before aspirating
10A(iii)	Cultures break apart when medium is added	Culture is very delicately assembled	Wait longer for more competent cultures to form before adding solutions. Leave the magnetic drive underneath the well plate while adding medium to preserve culture structure. Gently add medium to the wells
10B(ii)	Cannot see cultures in 96-well plate under microscope	Lid insert is blocking sight of cultures	Remove the lid insert in a sterile environment before imaging
10C(ii)	Magnet in the Teflon pen interacts with other magnets	Magnets are too close together	Keep separate magnetic pieces at a safe distance so that they do not interfere with each other
10C(iv)	The Teflon pen cannot reach all the way into the well to pick up 3D culture	The Teflon pen is too short to reach a particular 3D culture	Remove the magnetic drive and gently add medium to raise the 3D culture to pick up. The 3D culture should float for enough time to pick up
	Teflon pen cannot pick up the 3D culture	The magnetic drive is still under-neath the plate	Remove the magnetic drive from underneath the plate before picking up the 3D culture
10C(vi)	3D culture is not coming off the pen	3D culture is still attached to the pen	Dip the pen closer to the magnet repeatedly, or hover it over the magnet in a circular fashion. Also, tap the top of the pen to force the 3D culture off
		Magnet is still inside the pen	Remove the rod magnet from the Teflon pen before attempting to remove the 3D culture
16	Cultures break apart when solution is added	Culture is very delicately assembled	Leave magnetic drive underneath well plate while adding media to preserve culture structure. Gently add media to well
21	No fluorescence is seen when imaging stained 3D cultures	Primary or secondary antibody did not bind to antigen or primary, respectively	Check that solutions and incubations time used were correct. Wrap plates in foil after adding secondary antibody to prevent photobleaching

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● TIMING

Steps 1–4, incubation of cells with magnetic nanoparticles: working time 20 min, incubation time 5–16 h
Steps 5–9, creating 3D cultures with MLM in 96-well plates: 25 min
Step 10A, replacing medium in well plates with 3D cultures: 5 min

Step 10B, imaging 3D cultures in a well plate with an inverted microscope: 5 min
Step 10C, handling and transferring 3D structures with a Teflon pen: 5 min
Steps 11–14, fixing 3D cultures for IHC: working time 5 min, fixation time 15 min–4 h
Steps 15–21, whole-mount IHC of 3D cultures with fluorescence: working time 45 min, incubation time 4–16 h
Steps 22 and 23, transferring 3D cultures from plates to coverslips for microscopy with the Teflon pen: 5 min

ANTICIPATED RESULTS

Optimization of medium volume and cell number

When creating 3D cultures with the MLM, the medium volume and starting cell number must be optimized. We created 3D cultures of A549s, which were varied according to starting cell number (1,000–50,000) and medium volume (300–400 μl) in 24-well plates (**Fig. 2**). With increasing starting cell numbers, the 3D structures became larger. Cultures grown in 400 μl of medium were larger than those grown in 300 μl up to 4 d, but by day 7, there was no observable difference in size between the two volumes. These results demonstrate how 3D cultures can be optimized for starting cell number and medium volume.

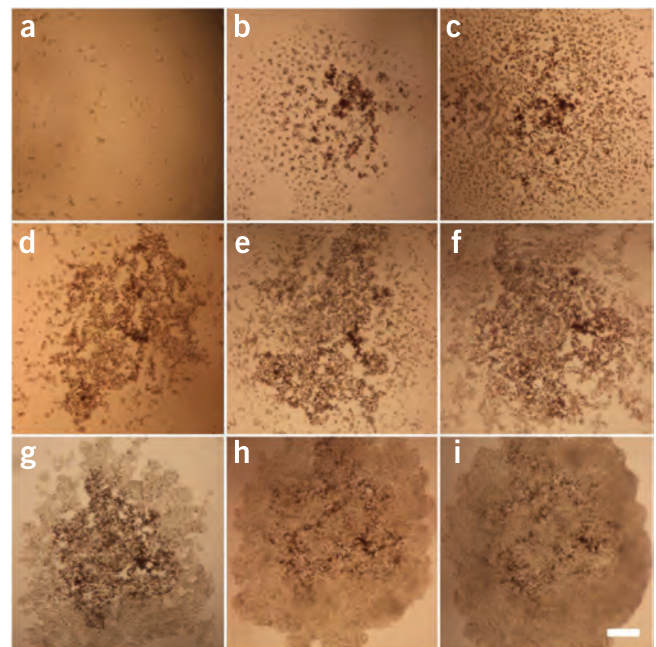

Figure 7 | Magnetically levitated 3D cultures of HepG2s. (**a**–**i**) After 0 min (**a**), 5 min (**b**), 15 min (**c**), 30 min (**d**), 45 min (**e**), 4 h (**f**), 24 h (**g**), 4 d (**h**) and 7 d (**i**). Scale bar, 250 μm.

Growth of 3D magnetically levitated cultures over time

To illustrate the typical results that are obtained using the MLM, we magnetically levitated HepG2 cells and imaged them at time points up to 7 d (**Fig. 7**). Immediately after the external application of a magnetic field, cells incubated with the magnetic nanoparticles were attracted toward the air-liquid interface and aggregated. Over the next day, the cells began to synthesize ECM and form competent structures. In addition, cells proliferated without the magnetic nanoparticles, and the nanoparticles appeared to cluster as the 3D culture grew around them after 1 d of levitation (**Fig. 7**). In subsequent days, these cultures became mature with minimal change in morphology, shape or nanoparticle density. These results show the typical progression of growth in magnetically levitated 3D cultures.

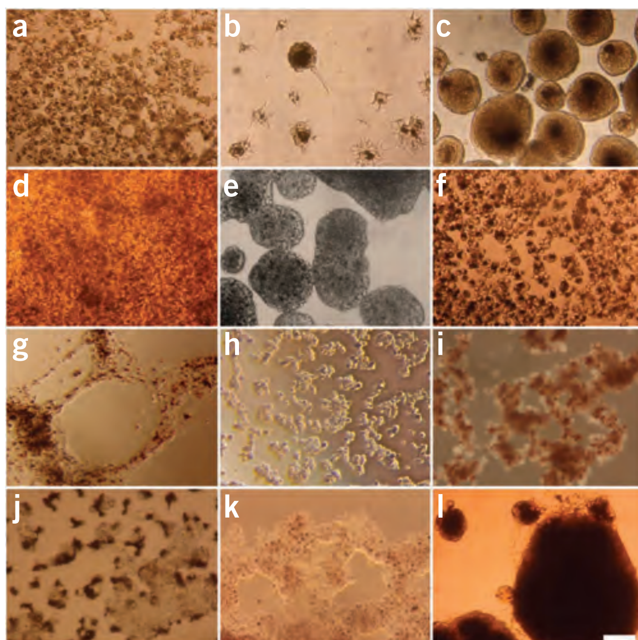

Figure 8 | Micrographs of magnetically levitated 3D cultures of various cell types. (**a**) HEK293; (**b**) human tracheal smooth muscle cells; (**c**) human pulmonary fibroblasts; (**d**) human glioblastoma; (**e**) H-4-II-E; (**f**) MDA-231; (**g**) human umbilical vein endothelial cells (HUVECs); (**h**) MCF-10A; (**i**) LNCaP; (**j**) HepG2; (**k**) A549; and (**l**) 3T3-L1. Scale bar, 100 μm.

Magnetic levitation of different cell types

When viewed under a microscope, magnetically levitated 3D cultures will vary among cell types in density of the nanoparticles, size and morphology (**Fig. 8**). For example, 3T3-L1 cells will tend to form small and dense clusters, whereas A549 cells will form less-dense and sparse structures. Common to 3D cultures of all cell types are the presence of the magnetic nanoparticles, as indicated by the brown color, and the dense packing of cells.

IHC of magnetically levitated 3D cultures

After culture, magnetically levitated 3D cultures can be analyzed with various analytical tools, such as IHC, similarly to 2D cultures or other 3D culture systems. For example, A549 cells were levitated with 175,000 cells and 400 μl of

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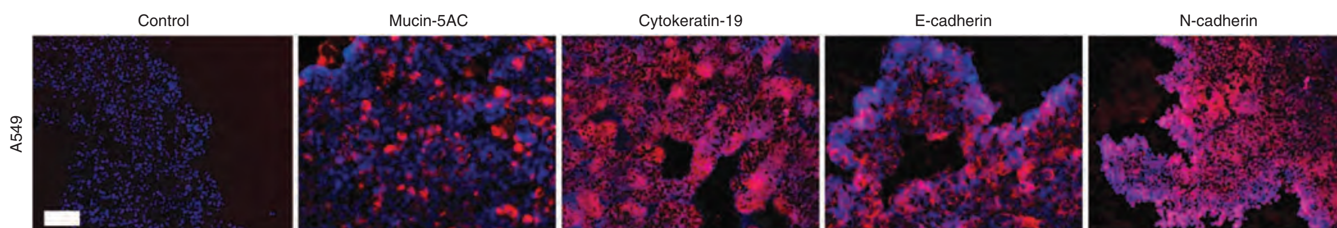

Figure 9 | Immunohistochemical staining patterns of 3D cultures of A549s for mucin-5AC (Abcam, cat. no. ab3649, 1:100 dilution), cytokeratin-19, (Abcam, cat. no. ab15463, 1:100 dilution), E-cadherin (Invitrogen, cat. no. 13-1700, 1:200 dilution), and N-cadherin (Invitrogen, cat. no. 33-3900, 1:100 dilution) after 2 d of culture. These 3D cultures were constructed with 175,000 cells per culture in 400 μl of medium. Positive staining patterns for mucin-5AC, cytokeratin-19 and E-cadherin verified epithelial phenotype and function, whereas N-cadherin demonstrated cell-cell interactions within the 3D culture. Scale bar, 100 μm.

medium in 24-well plates for 2 d before they were fixed in 4% (wt/vol) PFA. The cultures were then whole-mount stained for mucin-5AC, cytokeratin-19 and E-cadherin to verify epithelial cell phenotype and function and N-cadherin to view cell-cell interactions. The stained cultures were imaged using a confocal microscope. These cultures stained positively for all markers, indicating the maintenance of epithelial phenotype and function when cultured in 3D and demonstrating that the MLM maintains phenotype overall (**Fig. 9**). These results also demonstrate that immunofluorescence staining is possible in these cultures with no apparent interference from the magnetic nanoparticles.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS W.L.H. and D.M.T. contributed equally to this protocol by running the majority of experiments, gathering the bulk of the data presented in this protocol and preparing the manuscript. J.A.G. both helped in gathering data and images for this protocol. H.T. helped to write the manuscript. T.C.K. and G.R.S. invented and optimized the technique described in this protocol.

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